XP-002097835

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Tolerance of human fetal retinal pigment epithelium xenografts in monkey retina
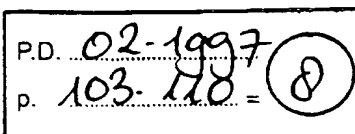
P.D. 02-1997
p. 103-110 = (8)

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Abstract ● Background: RPE transplantation offers the possibility of treating certain forms of retinal degeneration. Understanding how to optimize the surgical technique for performing RPE transplantation, especially in primates, is therefore of considerable interest. ● Methods: Fifteen patch RPE transplants were performed in six monkeys. The transplant sites were examined at follow-up by ophthalmoscopy, biomicroscopy, fluorescein angiography and histology. Foveal and peripheral retinal transplants were compared. ● Results: Human fetal RPE xenografts can survive without rejection for at least 6 months after transplantation in monkey retina. Such grafts form a basal lamina and make intimate contacts with the outer segments of the host. Both rods and cones retain a normal appearance when in contact with unrejected transplants. Rejection occurred in only 30% (3/10) of the peripheral but in 60% (3/5) of the foveal transplants. ● Conclusions: Cultured human fetal RPE patch transplants can survive and maintain local photoreceptor integrity for relatively long periods of time in monkey subretinal space without immunosuppression. Rejection, when it occurs, is more frequent near the fovea.

Introduction

Cultured human fetal retinal pigment epithelium (RPE) transplanted to the subretinal space of monkey retina can survive for at least 2–3 months without overt host/graft rejection, i.e. destruction of graft and cellular inflammation within or around the graft. Similar xenografts to rabbit retina are often rejected in a few weeks [28]. Adult RPE allografts survive well in the subretinal space of rat [18, 32] and rabbit [6] retina without immunosuppression, although in rats rejection can be provoked by subsequent sensitization [15]. In mice, however, there is evidence that RPE allografts are rejected [16], although photoreceptor allografts are well tolerated [11]. The long-term status of RPE transplants in the subretinal space is important because of attempts to use this approach to treat certain human forms of retinal degeneration [1, 2].

We have examined human fetal RPE transplants in monkey (*Macaca mulatta*) retina at 6 months after surgery and have compared parafoveal with more peripherally located (perimacular) subretinal transplant sites. The results indicate that only 30% of peripherally located xenografts overtly reject, whereas about 60% of foveally located ones do so. Semi-serial histological examination, including electron microscopy, reveals that unrejected RPE xenografts can retain a polarized epithelioid appearance and extend apical processes to contact host outer segments for at least 6 months without immunosuppression. An abstract describing some of these results has been published [12].
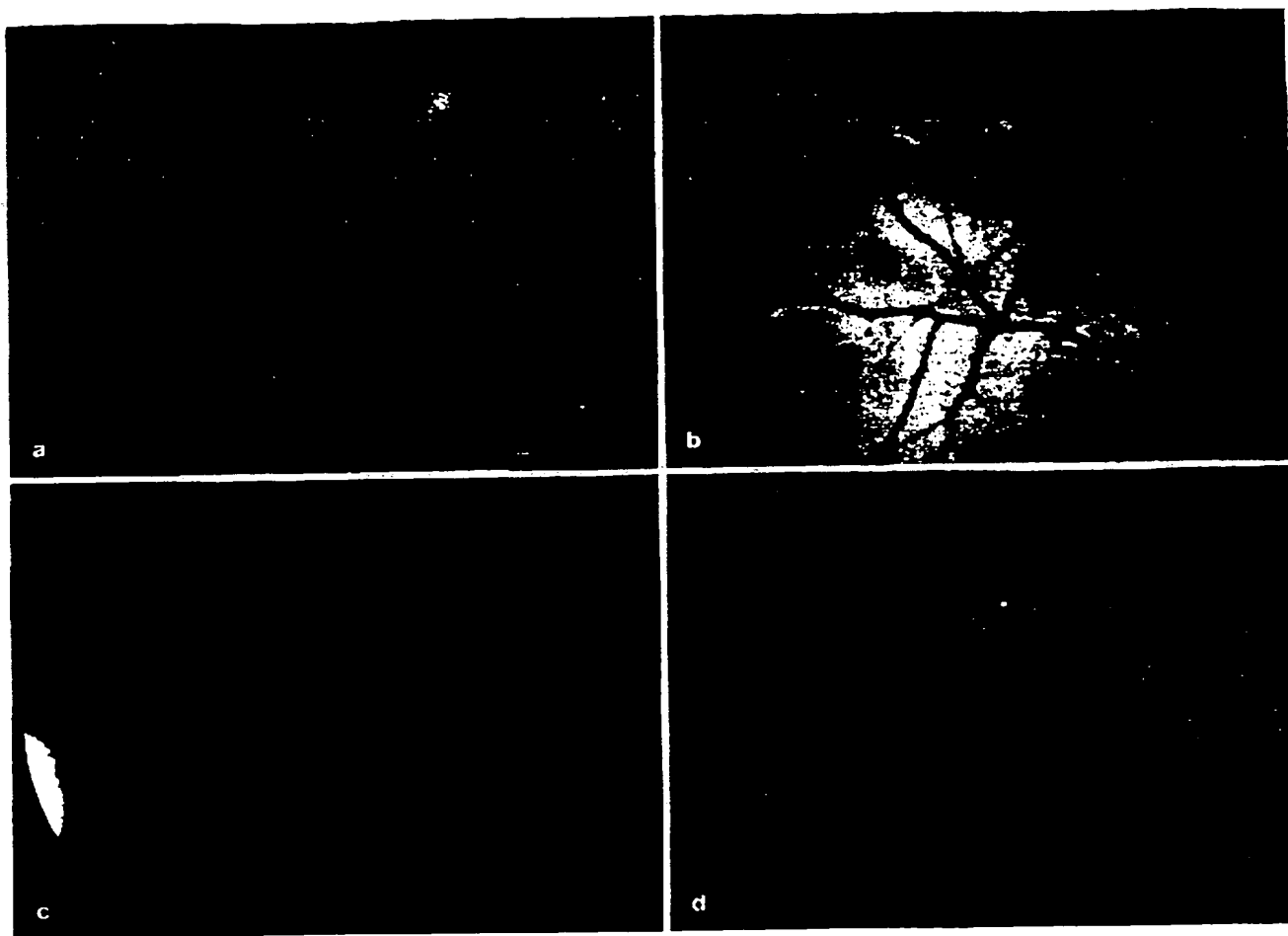
Fig. 1 Fundus photographs of foveal (a, c) and peripheral (b, d) human fetal RPE patch transplants in monkey retina at 6 months after surgery. The transplants in a and b show evidence of host/graft rejection, whereas those in c and d do not

Materials and methods

The methods for isolating and culturing human fetal RPE have been described previously [10, 28]. Fetal RPE (15–18 weeks gestational age) can be peeled off the choroid in monolayer sheets by gentle pulling with micro-forceps. The edges of each sheet are pressed into the surface of a culture plate with a needle tip. Within 4–6 days hexagonal RPE cells begin to grow out from the perimeter of the sheet to form a confluent monolayer. Contamination by other cells has not been observed and would be easy to detect because each patch is a transparent homogeneous mosaic of RPE cells [10].

Prior to transplantation surgery an area about 1 mm in diameter is cut out of an RPE monolayer, gently undermined with fluid and sucked into the barrel of a glass micropipette with an orifice di-ameter of 0.25–0.30 mm. The RPE patch folds when going through the orifice of the pipette and remains partially folded within its shank. Three pars plana ports in the eye of the recipient permit intraocular pressure control, illumination of the transplant site and, in the third port, introduction of an ocutome instrument and then a pipette for producing a bleb detachment and subsequently a transplant pipette. A local vitrectomy is performed at the selected transplantation site to facilitate production of the bleb detachment and re-entry of the same retinotomy with the transplant pipette. A saline-filled micropipette with a tip diameter of about 0.05 mm is used to create the detachment of the neural retina. The transplant pipette is then guided through this retinotomy and the transplant is slowly injected into the subretinal space, where it tends to unfold. This two-stage procedure allows more accurate placement of the transplant than the one-stage procedures described previously [28] because it reduces the amount of fluid injected with the transplant. After removal of the transplant pipette from the eye, the sclerotomy incisions and the overlying conjunctiva are closed with 8–0 Vicryl sutures. The neural retinal detachment reattaches over the transplant after several hours. For the surgery, which lasts 1 h or less, ketamine is administered subcutaneously (10 mg/kg), followed by intravenous sodium pentobarbital (Nembutal; 20 mg/kg/ h). The animals were examined at 1 week after surgery and thereafter at 2- to 3-week intervals using slit-lamp biomicroscopy, fun-
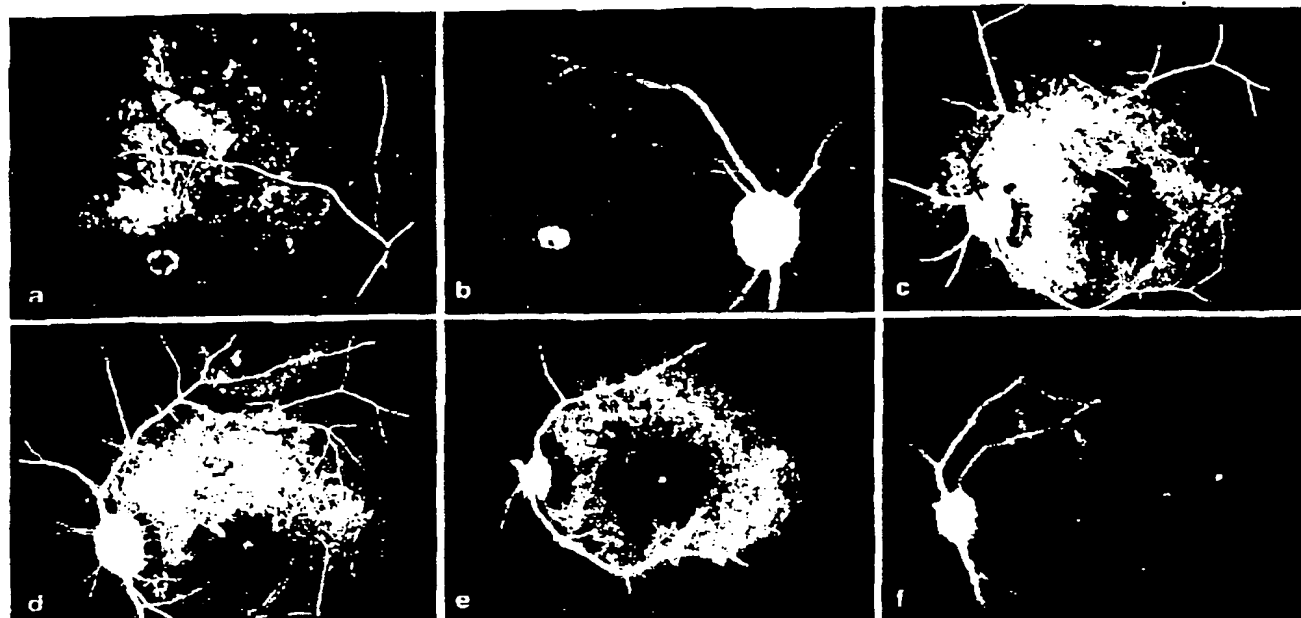
Fig. 2 Fluorescein angiograms showing both early (a, c, e) and late (b, d, f) phases of human fetal RPE transplants in monkey retina. The upper pair (a, b) show leakage, especially in the foveal transplant at 6 months after surgery. The middle and lower pairs show the same monkey at 6 weeks (c, d) and 6 months (e, f) after surgery respectively; there is slight evidence of leakage at 6 weeks but not at 6 months

dus photography and fluorescein angiography with ketamine, alone, as the anesthetic.

Fifteen transplants were performed in 6 monkeys. The monkeys were 1–2 years old at surgery. In two monkeys a transplant was placed near the fovea as well as at the periphery of each eye. The same donor tissue was used for transplants in the same animal. The two monkeys receiving binocular transplants recovered from surgery as rapidly as the others and behaved completely normal for the entire 6-month postoperative period. It should be realized that such transplants involve only microscopic areas of the retina [28]. The monkeys were killed at 6 months after surgery. Each eye was removed and punctured several times at the limbus to facilitate diffusion of the fixative. The eyes were immersed in a solution of 2% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline at pH 7.2 for at least 48–72 h. The eyes were then washed with buffered saline and dissected with the aid of a surgical microscope. The anterior segment, including the lens and vitreous, was removed and the transplant site identified. Each site was excised en bloc with adjacent surrounding tissue, post-fixed in 1% osmic acid, dehydrated and embedded in Epon. Semi-serial sections were taken through the transplant site extending from undetached retina on one side through the transplant to a similar area on the other side. The sections were examined to determine the status of the transplant and the adjacent host photoreceptors and the presence of inflammatory cells in the retina and choroid. In certain areas ultrathin sections were also examined by electron microscopy.

We measured the width of four peripheral and three foveal transplants including the host RPE layer, the width of the inner and outer segments of the adjacent photoreceptors and the width of the

outer nuclear layer; in addition, we measured the width of the RPE layer and photoreceptor dimensions in areas of detached retina which had no transplant, as well as in adjacent undetached retina. We have also included measurements of two transplants, at 2 months after surgery, which were described qualitatively in a previous publication [28] but are relevant here.

Informed consent and institutional approvals were obtained for the use of all of the human donor tissue. The tenets of the Declaration of Helsinki on human experimentation and the ARVO *Statement for the Use of Animals in Ophthalmic and Vision Research* were followed.

Results

Fundus photographs of two foveally and two more peripherally located human fetal RPE transplants in monkey retina at 6 months after surgery are illustrated in Fig. 1. The upper photographs show transplants that have evidence of host/graft rejection, more obvious in the foveal (a) than in the peripheral one (b); the other transplants (c,d) display no evidence of rejection. The rejected transplants become smaller with time and are surrounded by a light ring of retina. The unrejected transplants do not change with time. There is a discoloration in the fovea, suggesting RPE atrophy, close to both parafoveal RPE transplants. The peripheral transplants are surrounded by a circular area of RPE lightening demarcating the extent of the original bleb detachment. This is not obvious in the parafoveal transplants.

The parafoveal transplant with funduscopic evidence of rejection (Figs. 1a and 2a) shows fluorescein leakage most apparent in the late frame of the angiogram (Fig. 2b). The more peripheral transplant shows slight
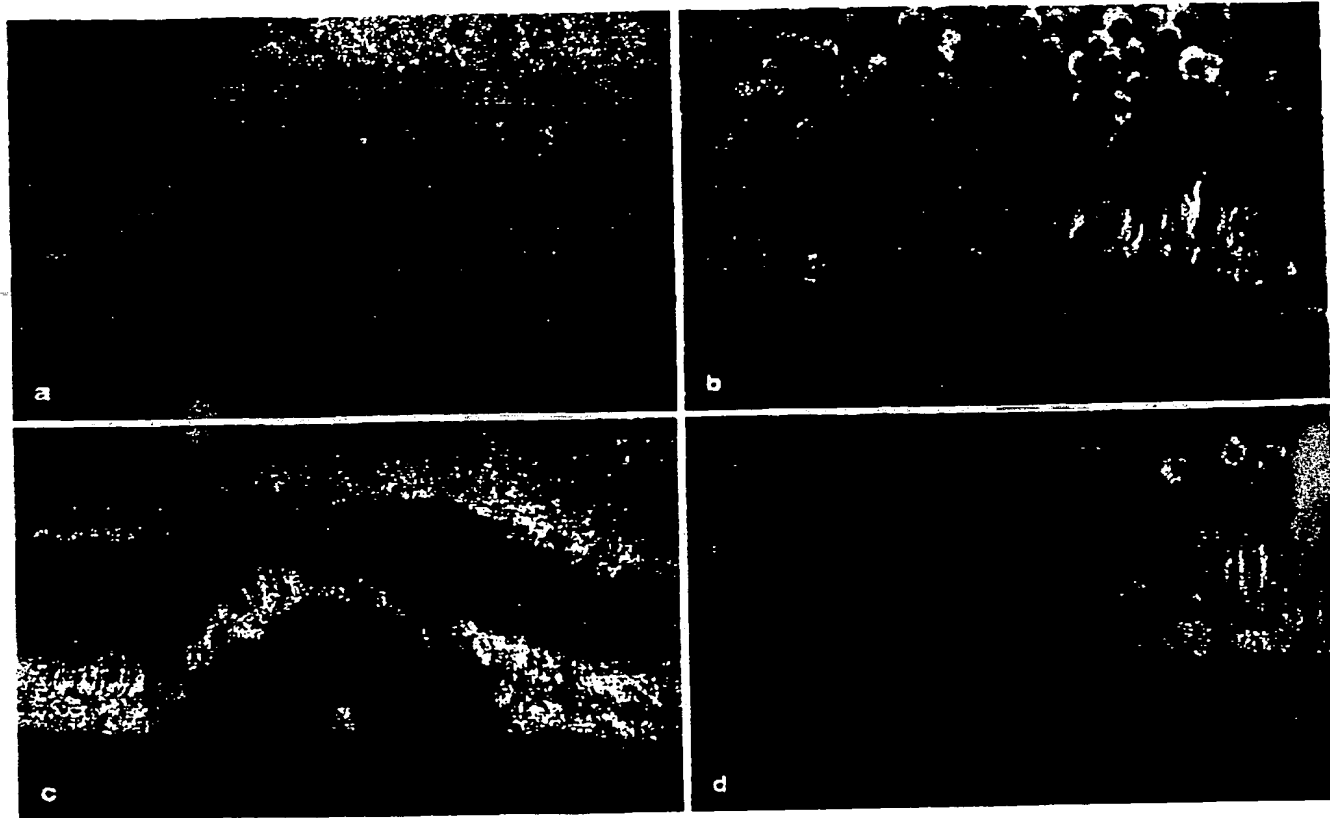
Fig. 3 Light micrographs of foveal (a, c) and peripheral (b, d) human fetal RPE patch transplants in monkey retina at 6 months after surgery Foveal transplant a shows evidence of host/graft rejection

leakage at its upper border but had no evidence of rejection histologically.

Fluorescein angiograms of the two unrejected transplants of Fig. 1c,d are shown at 6 weeks (Fig. 2c,d) and at 6 months (Fig. 2e,f) after transplantation. At 6 weeks there is slight staining along the upper pole of both transplants which has disappeared at 6 months. There is no change in the size of these transplants over time (Fig. 2c–f).

Figure 3 shows light micrographs of four different RPE xenografts at 6 months after surgery, two in the foveal area (a,c) and two located more peripherally (b,d). One of the foveal ones shows evidence of rejection (Fig. 3a). Little of the original RPE transplant is present, and fibroblast-like cells separate the neural retina from the few remaining pigmented transplant cells. The photoreceptor layer adjacent to the transplant has degenerated. The host RPE layer and the choriocapillaris directly under the transplant remain intact. The destruction is very local. There is no evidence of hemorrhage.

The other foveal transplant (Fig. 3c) shows no evidence of rejection at 6 months after surgery. It sits on the host RPE layer and contacts outer segments of neighboring photoreceptors. It is multilayered and exhibits a cross-sectional profile that reflects its funduscopic appearance (Figs. 1c, 2c–f). Figure 4 shows a magnified view of this transplant where its apical surface is in close contact with host outer segments.

Figure 3b shows a more peripherally placed transplant in the same retina where a foveal transplant had evidence of rejection. This peripheral transplant has no evidence of rejection. It appears as a pigmented monolayer sitting on the host RPE. Well-oriented outer segments, of both rod and cones, are contacting its apical surface. There is no evidence of inflammation in or around the transplant; the adjacent choriocapillaris appears normal.

Figure 3d shows a peripherally placed transplant (Figs. 1d, 2c–f) with no evidence of rejection at 6 months after transplantation. This transplant is multilayered, especially toward its center. It has displaced the host RPE layer from Bruch's membrane. The photoreceptor outer segments in contact with the apical surface of the transplant are well oriented and have relatively long outer segments. An electron microscopic view of these contacts

Fig. 4 Light micrograph of the foveal transplant shown in Fig. 3c. Outer segments of photoreceptors are in close contact with the upper layer of the transplant

Fig. 5 Electron micrographs of the human RPE xenograft of Fig. 3d, showing a multilayered transplant (a) resting on Bruch's membrane with each layer separated by a Bruch's membrane-like extracellular structure. The RPE in the uppermost layer has apical processes extending to the photoreceptors. Some of these outer segments are in close contact with these apical processes (b). There is a basal lamina (c, *large arrows* at *top left*) next to RPE layers facing each other in the transplant. The basal surface of the transplant facing the choriocapillaris has a basal lamina (*large arrow, bottom*) and a few infoldings (*small arrow*). a ×2450, b ×18000, c ×18000

shows that there are fine processes extending from the apical surface of the transplant which embrace the host outer segments (Fig. 5a, b). The basalmost layer of the transplant is sitting on Bruch's membrane and has a distinct basal lamina (Fig. 5c). There are infoldings along the basal membrane at some areas of the transplant. The multilayers of the transplant are separated from each other by an extracellular matrix that resembles Bruch's membrane. The transplanted RPE forms a basal lamina on both sides of this extracellular structure (Fig. 5a, c) There is no evidence of inflammation in the transplant or the adjacent retina and choroid.

In order to assess the effects of such transplants on the host photoreceptors, we measured the widths of the outer nuclear layer and the inner and outer segments of the photoreceptors over the transplant and compared them to the widths of the transplant and the host RPE layer. These measurements were made at the center and along the edge of the transplant, in the neighboring detached retina not receiving a transplant and in the control retina adjacent to the detachment.

Such measurements for three tolerated peripheral transplants at 6 months and one at 2 months after surgery are illustrated in Fig. 6. A slight decrease in the widths of the outer nuclear layer and the outer and inner segments of the photoreceptors is produced by the detachment alone. The transplant produces only a further slight decrease of photoreceptor width. This decrease does not appear to depend much on the width of the transplant. The photoreceptors over thicker areas of the transplant appear similar to those over thinner areas. The differences at 2 months resemble those at 6 months after transplantation.

Similar measurements for three parafoveally tolerated transplants are illustrated in Fig. 7. Transplants in or near
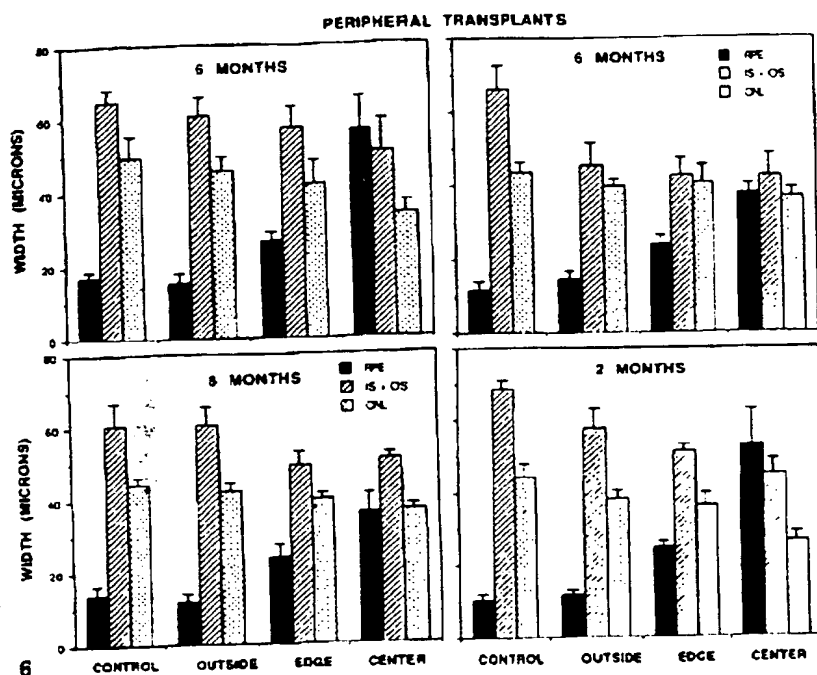
Fig. 6 Measurements of the widths of the RPE, the inner and outer segments (*IS+OS*) and the outer nuclear layer (*ONL*) in neighboring undetached retina (*control*), within the bleb detachment but without any RPE transplant (*outside*), at the edge of the RPE transplant (*edge*) and in the center of the RPE transplant (*center*) for peripheral sites: three measurements at 6 months and one at 2 months after surgery. Each measurement represents the average of approximately the same point in 6–20 consecutive sections. The *vertical lines* show the standard deviations of these measurements. The *ordinate* represents the width in micrometers. At the detachment the width includes both the host monkey RPE and the human RPE patch transplant; in some cases no host RPE was found

Fig. 7 The same measurements as described in Fig. 6 but of transplants placed in or near the foveal area. The middle graph represents the foveal transplant of Fig. 1c

the fovea tend to be more multilayered and therefore thicker than those placed more peripherally. Otherwise the effects of transplantation on the adjacent photoreceptors are similar for both peripheral and foveal transplants.

Ten transplants of 6 months' duration were located in more peripheral and five were located in parafoveal retina. Only three of the ten peripheral transplants had evidence of rejection, i.e. complete or partial destruction of the transplant, cellular inflammation and degeneration of the adjacent photoreceptor layer. Three of the five foveally placed transplants, however, showed evidence of rejection. Rejection was found in one and not in the other eye of the same monkey and in the fovea but not in the periphery of the same retina.
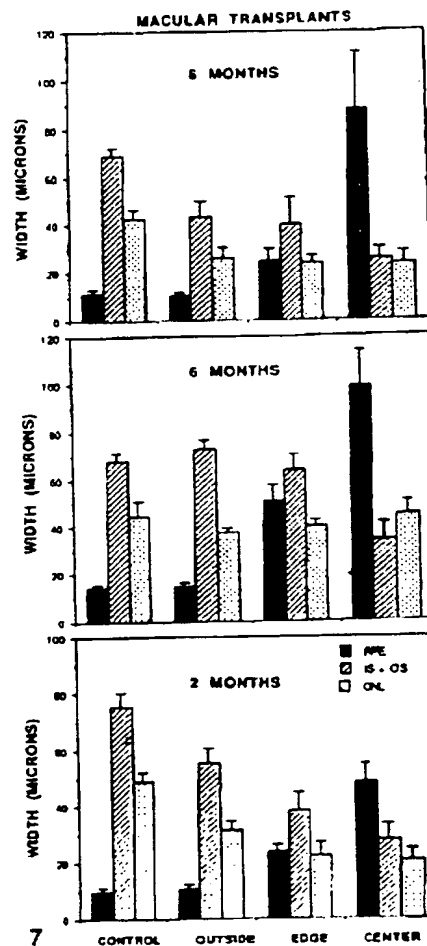
Peripheral detachments produced a mottling of the retina that covered the original bleb detachment (Fig. 1); this was also seen as multiple fine window-like defects by fluorescein angiography (Fig. 2). This was not apparent in the foveal detachments. We have found that vacuoles appear in some RPE cells in the area of peripheral bleb detachments which we considered responsible for window-like defects [28]. In the present experiments we have observed that there are fewer melanin granules in the host RPE in areas of the peripheral retinal detachments, another factor that may be responsible for window-like defects after detachment and/or RPE transplantation.

Discussion

Cultured human fetal RPE xenografts can survive in the subretinal space of monkeys for at least 6 months without

signs of host/graft rejection. Some such xenografts, especially those at or close to the fovea, are rejected. Rejection has been based on graft fragmentation, cellular inflammation and degeneration of the adjacent photoreceptors. The survival of xenografts within the central nervous system is not unprecedented. Embryonic mouse retinal xenografts survive and develop within rat brain [27].

It seems unlikely that what we have called rejection is graft failure due to unsatisfactory surgery, because the degenerative and inflammatory changes are confined to the transplant itself. They are absent immediately adjacent to the transplant; even the local host RPE may be undisturbed. There is also no evidence of hemorrhage, which often reflects surgical trauma. We have previously described such traumatic hemorrhage around RPE transplants in rats due to microelectrode recordings [32]. We have experienced similar rejection, both in retinal and conjunctival placed xenografts [28] and its appearance resembles what we have described here. Such rejection can be eliminated by cyclosporine immunosuppression [29]. Therefore we believe that rejection occurs in only a fraction of human RPE xenografts to monkey retina. Supplementary methods may be used in the future to monitor the development of cellular and/or humoral signs of rejection by sampling blood or tissue lymphocytes or determining serum antibodies to the cultured donor RPE, although unequivocal standards for host/graft rejection are not easy to establish [23].

Why rejection appears to be more likely with foveal transplants is not clear. Perhaps our sample is too small. Perhaps either a greater choriocapillary bed in the foveal area provides more opportunity for the immune system to detect foreign tissue or the surgery required to place a transplant in the subfoveal space provokes more inflammation and consequently more potential immune surveillance. There is evidence that bleb detachments of the neural retina in the foveal area have different consequences than those produced more peripherally. The latter lead to numerous window defects in the host RPE, demarcating the entire detachment. This does not seem to occur near the fovea, possibly because the blebs are smaller and the local host RPE is obscured by the transplant. This smaller volume may result in higher local pressure changes within the area where the transplant is placed. This may cause local trauma that induces blood cells to enter the transplant site shortly after surgery, which subsequently leads to rejection.

Equally surprising is the evidence of rejection of a transplant in one but not in another area of the subretinal space in the same animal. In a previous paper we described inflammatory cells in the choroid adjacent to a human RPE xenograft at 2 months after surgery, which was the only one in these experiments that was close to the fovea [28]. We argued that this was not a sign of rejection because there was no evidence of inflammation

around similar transplants from the same donor which were located more peripherally in the same retina. We were assuming that if rejection occurred, all transplants in that animal derived from similar donors would be rejected. Now, with more experience with subretinal xenografts, we must reconsider this assumption.

Since the pioneering work of Medawar [21], grafts within an immunologically privileged site such as the anterior chamber are known to be rejected if the hosts are systemically sensitized by peripheral immunization. There are examples, however, where this generalization is disobeyed. Some rats will not reject embryonic neural allografts transplanted to the brain even though the host rats are systemically immunized to the donor tissue [14, 24].

The unusual behavior of subretinal transplants may reflect the uniqueness of this immunologically privileged site [16, 17, 22, 28, 31]. MHC class II-positive cells are not present in the normal retina, even after the provocation of endotoxin induced uveitis [33]. γ-Interferon, a potent inducer of MHC antigen expression, does not appear to be expressed in the retina [5].

Multilayered RPE transplants were virtually as effective as monolayer transplants for maintaining the integrity of the neighboring photoreceptors. In some cases the photoreceptors were four or five times further away from the choriocapillaris than is normally the case. Detachment of the neural retina causes degeneration of photoreceptor outer segments and subsequently inner segments [3, 4, 7, 8, 20]. The fact that the photoreceptors do not degenerate adjacent to these multilayered transplants implies that photoreceptor degeneration from neural retinal detachment is due more to loss of contact with the RPE layer than to separation from the choriocapillaris. It also implies that removal of the host RPE may not be necessary for obtaining a possible therapeutic effect from an overlying RPE transplant. In this regard it is noteworthy that in some cases the host RPE under the transplant appears to be atrophic (see Fig. 3b, c) and in other cases the transplanted fetal RPE has insinuated itself directly onto Bruch's membrane (Fig. 3d). There may be some competition between the fetal and host RPE, or perhaps more direct contact with the photoreceptor layer favors the survival of the transplant. More research will be needed to resolve this issue.

The fact that RPE xenografts can survive for long periods of time in the subretinal space and exist in direct contact with relatively healthy looking photoreceptors, both rods and cones, suggests that human-to-human RPE allografts should do as well or better.

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